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DRUG INTERFERENCE WITH THE TRIHYDROXYINDOLE METHOD FOR FREE CAT--ETC(U)
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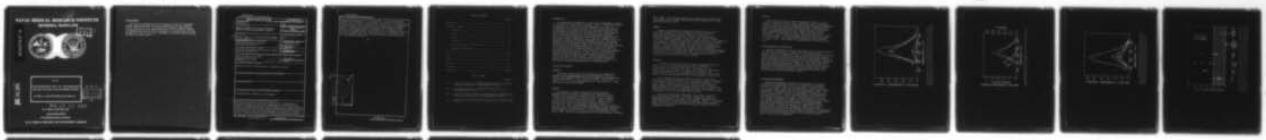

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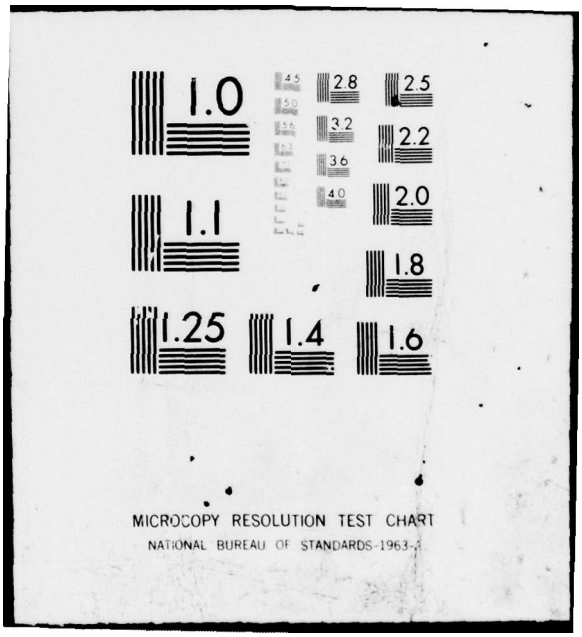
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DRUG INTERFERENCE WITH THE TRIHYDROXINDOLE METHOD FOR FREE CATECHOLAMINES IN URINE

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Thirty-five of 108 drugs tested may interfere with the trihydroxyindole fluorometric method for urinary free catecholamines. Screening for interference was accomplished by testing the total daily dose of a drug or portion thereof for possible fluorescence. Further information was obtained by adding a similar dose for each of 16 interfering drugs to 150 ml of fresh, pooled urine containing 67 ng/ml each of norepinephrine and epinephrine. Additional studies of seven interfering drugs were conducted using healthy males and

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assaying catecholamines from 4-h urine specimens. Four possible modes of interference were observed: (1) substances which reacted to form fluorescent products, such as thiamine hydrochloride and ascorbic acid; (2) substances with native fluorescence, such as riboflavin; (3) substances which are intensely colored in solution and interfered with fluorescent excitation or emission spectra, such as phenazopyridine hydrochloride; and (4) substances which inhibited catecholamine extraction onto alumina, such as methenamine mandelate. This work has expanded upon data previously available in identifying drugs which may spuriously alter urinary free catecholamine excretion measured by the trihydroxyindole procedure.

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INTRODUCTION

The quantitative determination of urinary free catecholamine excretion has been studied in both health and disease. Used to establish the presence of chromaffin cell tumors such as pheochromocytoma (1), studies of catecholamine excretion have also been examined in such diverse diseases as hypertension (2), diabetes mellitus (3), and muscular dystrophy (4). Catecholamine excretion has also been studied in acute stress situations such as myocardial infarction (5), thermal injury (6), and physical exertion under varying work loads (7) and environmental conditions (8,9). While recent work has improved the sensitivity of the trihydroxyindole fluorometric method by optimizing various analytical conditions such as pH and optimal excitation and emission wavelengths (10-16), corresponding emphasis has not been directed towards identifying substances which may interfere with the fluorometric method (17). Previous studies have discussed the specificity of the trihydroxyindole method (18,19) and identified several medications which spuriously elevate results due to native or developed fluorescence (20-24). Other drugs such as methenamine mandelate may yield falsely decreased results (25). Ideally, no drug should be administered during evaluation of catecholamine excretion; yet such circumstances are not always possible. Increased knowledge of drugs that may interfere with the trihydroxyindole fluorometric method is important to avoid distortion of results and diagnostic confusion (26). This study has examined 108 substances for such interference.

MATERIALS AND METHODS

Apparatus

Fluorescence spectra and relative fluorescence measurements were obtained using an Aminco-Bowman spectrophotofluorometer with models P416-992 xenon lamp and 10-222 solid state photomultiplier microphotometer (American Instrument Co., Silver Spring, MD 20910). "Biomed" mini-columns (1 cm i.d. X 18 cm; New England Nuclear, Worcester, Mass. 01608) were used for elution from alumina.

Reagents

Glass-distilled, de-ionized water was used for solutions. Standard solutions (1 mg/ml) of epinephrine and norepinephrine were prepared with 0.01 mol/liter hydrochloric acid. Working standards (1 Hg/ml) were prepared prior to use by diluting 0.1 ml of standard to 100 ml with 0.01 mol/liter hydrochloric acid. Standard solutions were stored at 4°C for no longer than two weeks. Alumina (neutral, Brockman activity grade 1) was obtained from Sigma Chemical Co., St. Louis, Mo. 63178. Norepinephrine, epinephrine, caffeine, salicylic acid, uric acid, riboflavin and pyridoxine hydrochloride were obtained from Aldrich Chemical Co., Milwaukee, Wisc. 53233; trimethylamine, thiamine hydrochloride and nicotinic acid were obtained from Calbiochem, La Jolla, Calif. 92037. Minoxidil was supplied as a gift by Upjohn Company, Kalamazoo,

Mich. 49001. The remaining compounds were pharmaceuticals obtained from the Pharmacy Service, National Naval Medical Center, Bethesda, Md. 20014. Drugs were used as received.

Samples

Samples for screening were prepared by dissolving part or all of a total daily adult dose of a given compound in 150 ml of de-ionized, distilled water which was adjusted to pH 3 with 12 mol/liter hydrochloric acid and then analyzed. Further studies on interfering drugs were conducted by adding a similar dose of the specific substance to 150 ml of fresh, pooled urine adjusted to contain 10 μg each of norepinephrine and epinephrine. Additional studies were conducted with healty men taking a specified dose of a given compound for seven days suring routine daily activity. Dietary substances known to interfere with the trihydroxyindole method were avoided. Four-hour urine specimens (0800 to 1200h) were collected on the last two days of drug administration, and analyzed immediately or stored at -35°C for no longer than 5 days. Two 4-h urines (0800 to 1200h) collected from each individual prior to drug administration and during routine daily activity served as control specimens.

Procedure

Fluorometric analysis of samples followed the procedure described in detail by Crout (27), including steps for alumina preparation. Catecholamines were extracted onto alumina from samples at pH 8.4 and eluted with 0.2 mol/liter acetic acid. Catecholamines in the alumina column eluate were converted to trihydroxyindole derivatives and assayed fluorometrically. *In vivo* studies were corrected to 100% recovery based on recovery determined for control solutions analyzed simultaneously.

After correcting for blank fluorescence if present, the relative intensity of observed fluorescence for an interfering substance was compared to fluorescence obtained for the 1 μg/ml norepinephrine standard. This ratio defines a relative interference by percent and cannot be used to predict fluorescent interference *in vivo*. Substances were considered to exhibit moderate interference if this ratio was less than 10 percent of the fluorescence observed for the norepinephrine standard, and to exhibit strong interference if the ratio was greater than 10 percent.

Since the trihydroxyindole fluorophores are unstable, consistent blank preparation is necessary. The blank is formed by allowing the fluorescence developed by the sample to fade through oxidation of trihydroxyindole derivatives to non-fluorescing compounds. Therefore, an abnormal blank was defined as a blank differing from the norepinephrine standard blank, which is usually 10 percent of the fluorescent intensity of the norepinephrine standard (27).

RESULTS

The results for initial testing are presented in Tables 1, 2 and 3. For the 73 substances listed in Table 1 fluorescent spectra differed only slightly or not at all from the corresponding blank, and thus no interference would be expected with the trihydroxyindole procedure. Substances which exhibited fluorescent activity at the excitation and emission wavelengths used in the dihydroxyindole method for dopamine (28) are also noted. Twenty compounds are presented in Table 2 which exhibited moderate interference. Representative fluorescent spectra for drugs of this category are given in Figure 1. Fifteen substances exhibiting strong interference are listed in Table 3 with representative fluorescent spectra presented in Figures 2 and 3. The presence of an abnormal blank is noted in Tables 2 and 3.

Drug Interference in Pooled Urine

Apparent catecholamine recoveries from pooled urine for sixteen substances and three drug combinations or regimens that might be encountered in clinical practice are presented in Table 4. For substances such as triamterene, quinidine sulfate and chlorpromazine hydrochloride which yielded abnormal blanks, epinephrine values were not consistent with norepinephrine recoveries and both spuriously elevated and decreased results were obtained. Interfering drugs with normal blanks, such as ascorbic acid and thiamine hydrochlorede, gave falsely elevated catecholamine recoveries. Other substances such as phenazopyridine hydrochloried and rifampin form intensely colored solutions druing the trihydroxyindole procedure. These drugs have strong visible absorption at or near the fluorescent excitation maximum (410 nm) which greatly reduces fluorescent emission (Figure 3).

In Vivo Drug Interference

Apparent norepinephrine excretions in the presence of some common medications each administered orally to five healthy men are presented in Figure 4 with control data for each test subject. Reference values for 4-h urines (0800 - 1200 h) for 25 determinations from 11 other healthy male subjects during routine daily activity are presented as mean ± 2 SD (shaded area). Each symbol in Figure 4 represents an average of two 4-h urine collections (0800 - 1200 h); the mean difference for norepinephrine excretion between the two control values obtained for each of the six participating individuals was 8 ng/min. With administration of ascorbic acid, thiamine hydrochloride or riboflavin, norepinephrine excretion appears to be spuriously elevated; mentenamine mandelate or phenazopyridine hydrochloride administration resulted in falsely decreased norepinephrine excretion. Triamterene (100 mg/d) was administered to two test subjects (AG,LM) using the same experimental protocol and apparent norepinephrine excretion was 75 and 25 ng/min, respectively. Two hypertensive males on chronic methyldopa and diuretic therapy had apparent norepinephrine excretions of 290 and 445 ng/min based on two 4-h urine collections (0800 - 1200 h) for each individual.

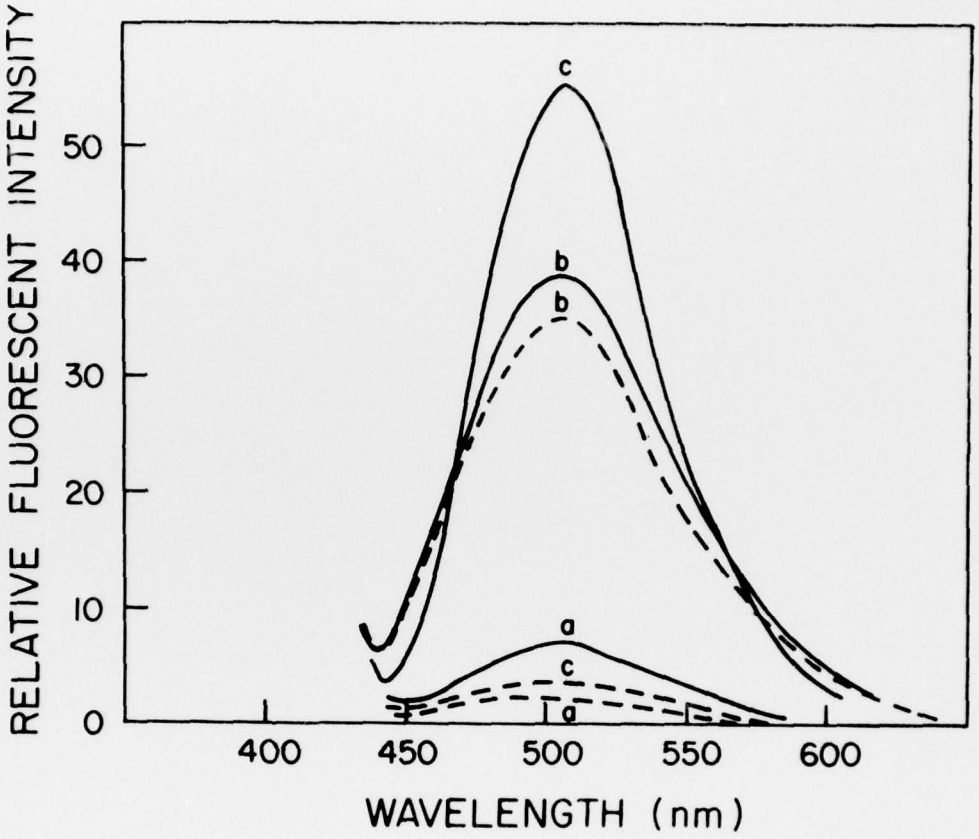

Fig. 1. Fluorescent Spectra for Thiamine Hydrochloride and Tetracycline Hydrochloride During the Trihydroxyindole Procedure. *Fifty mg of thiamine hydrochloride* (curve a), 250 mg of tetracycline hydrochloride (curve b), or 0.2 ml of 1 µg/ml norepinephrine standard (curve c) were added to 150 ml of acidified, distilled water and extracted with alumina at pH 8.4. Two hundred µl of the respective column eluates were analyzed by the trihydroxyindole procedure. Blanks are indicated by dashed lines. Excitation wavelength was 410 nm.

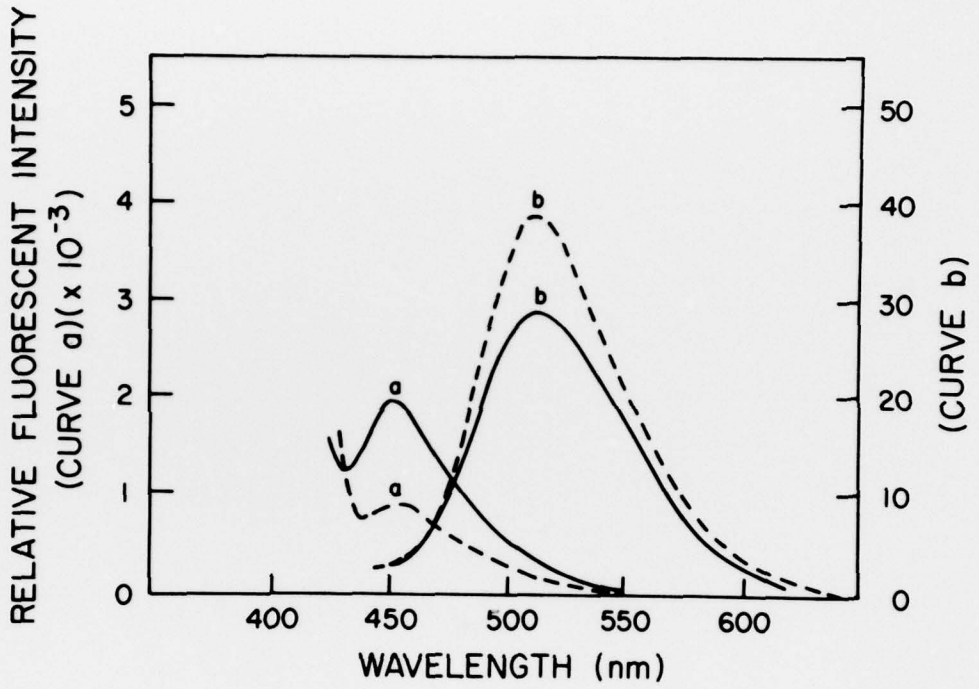

Fig. 2. Fluorescent Spectra for Triamterene and Riboflavin in the Trihydroxyindole Procedure. One hundred mg of triamterene (curve a) or 50 mg of riboflavin (curve b) were treated as described in Figure 1. Dashed lines represent sample blanks.

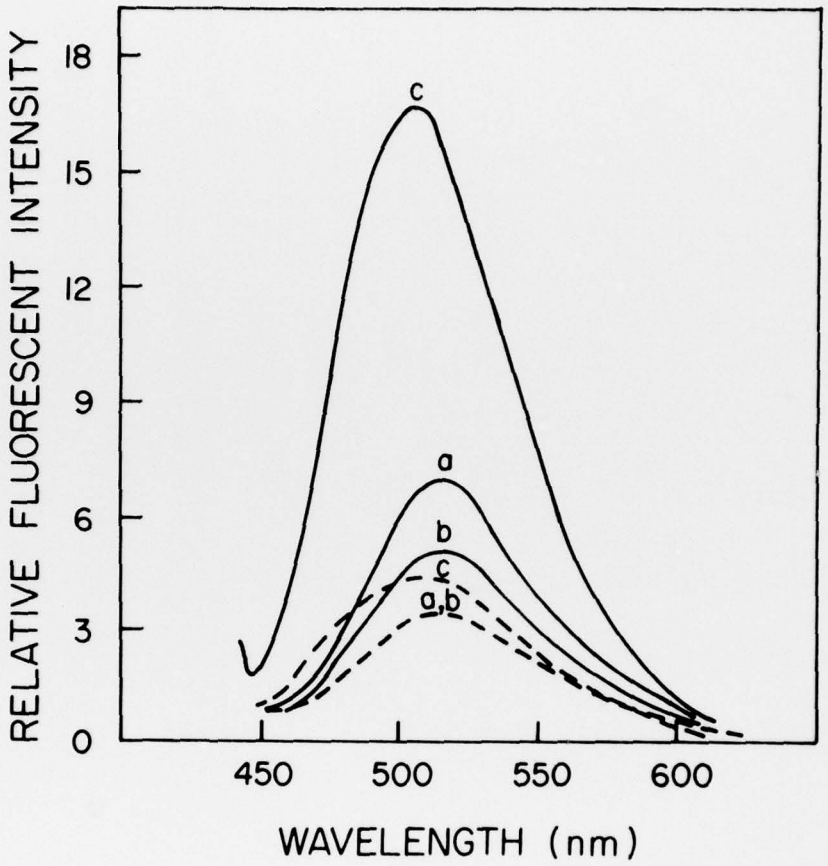

Fig. 3. Fluorescent Spectra Obtained for Phenazopyridine Hydrochloride in Pooled Urine Analyzed by the Trihydroxyindole Procedure Curves a and b are the respective fluorescent spectra for 200 µl and 100 µl aliquots of the column eluate obtained from alumina extraction of 150 ml of pooled urine containing 67 ng/ml each of norepinephrine and epinephrine to which 150 mg of phenazopyridine hydrochloride had been added. Curve c is the spectrum obtained for a 100 µl aliquot of a 1.0 µg/ml norepinephrine standard. Sample blanks are indicated by dashed lines; excitation wavelength was 410 nm.

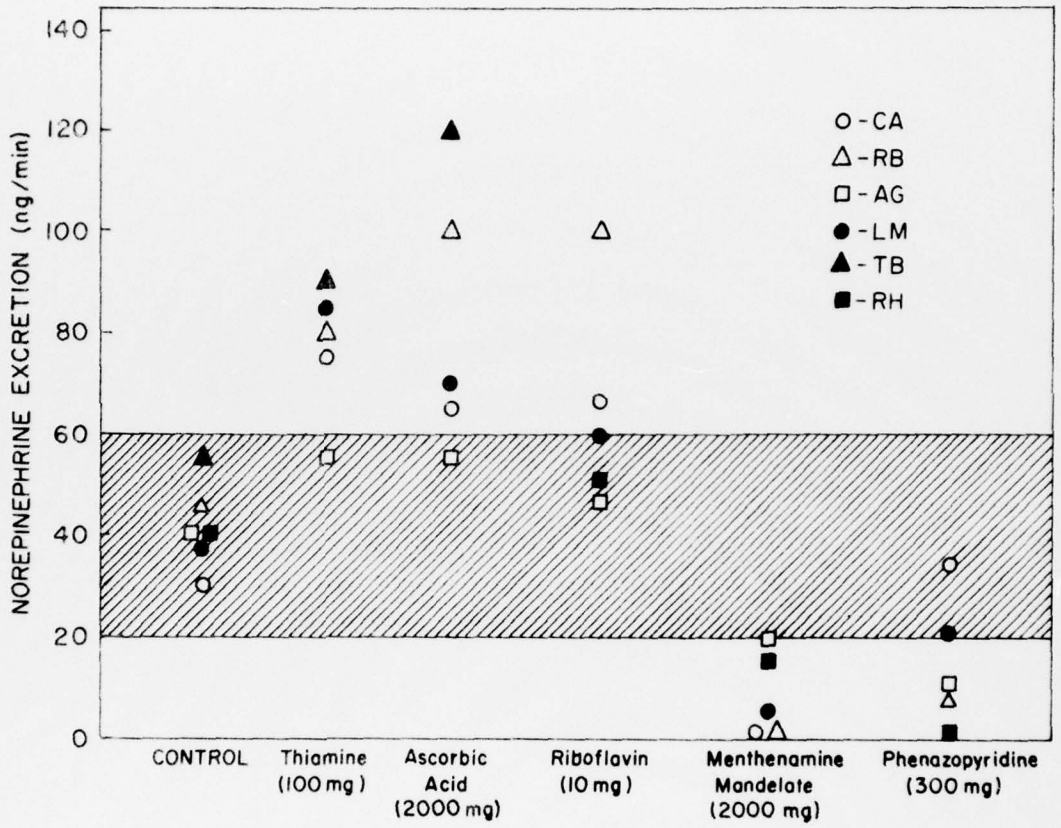

Fig. 4. Some Selected Drug Interferences In Vivo with the Trihydroxindole Method. Apparent norepinephrine excretion in ng/min was determined for five healthy males taking each of five selected medications for seven days during routine activity. Each symbol represents an individual and is the mean value from two 4-h urine specimens (0800 to 1200 h) collected prior to (control), and on the last two days of each drug administration. The shaded area represents the mean ±2 SD (40 ± 20 ng/min) for 25 determinations of norepinephrine from 11 other healthy males under similar control conditions. The total dose of a drug is expressed in parentheses with administration by mouth as follows: thiamine hydrochloride 100 mg daily in AM, ascorbic acid 500 mg every 6 h, riboflavin 10 mg daily in AM, methenamine mandelate 500 mg every 6 h, and phenazopyridine hydrochloride 100 mg every 8 h.

TABLE 1. Substances Exhibiting No Fluorescence During *In Vitro* Testing with Trihydroxyindole Procedure[a]

Acetaminophen (325)
Acetazolamide (500)
Acetohexamide (250)
Acetylsalicylic acid[b] (325)
Actifed [Burroughs Wellcome]:
- Pseudoephedrine hydrochloride (60)
- Tripolidine hydrochloride (2.5)

Allopurinol (50)
Aminophylline (200)
Anacin [Whitehall]:
- Acetylsalicylic acid (800)
- Caffeine (64)

APC[b]
- Acetylsalicylic acid (230)
- Caffeine (32)
- Phenacetin (170)

Biotin (5.0)
Diazepam (10)
Diazoxide (300)
Dimenhydrinate (25)
Diphenylhydantoin sodium (100)
Ephedrine sulfate (50)
Ethacrynic acid (100)

Ethanol (80)
Excedrin [Bristol-Myers]:
- Acetaminophen (97)
- Acetylsalicylic acid (194)
- Caffeine (65)
- Salicylamide (129)

Fenfluramine hydrochloride (20)
Flurazepam hydrochloride (30)
Furosemide (40)
Haloperidol (1.0)

Hydralazine hydrochloride (50)
Hydrochlorothiazide (100)
Hydroxyzine hydrochloride (25)

Indomethacin (25)
Insulin isophane (40 units)
Insulin protamine zinc (40 units)
Levothroxine sodium (0.05)

Brondecon [Warner/Chilcott]:
- Glyceryl guaiacolate (400)
- Oxtriphylline (800)

Caffeine (200)
Chlordiazepoxide hydrochloride (10)
Chlorpheniramine maleate (4.0)
Chlorpropamide (125)
Choline chloride (10)
Chromolyn sodium (20)
Clofibrate (500)
Clonidine hydrochloride (0.2)
Colchicine (0.325)
Contac [Menley & James]:
- Beoladonna alkaloids (0.2)
- Chlorpheniramine maleate (4.0)
- Phenylpropanolamine hydrochloride (50)

Dextroamphetamine sulfate (5.0)
Lomotil [Searle & Co.]:
- Atropine sulfate (0.05)
- Diphenoxylate hydrochloride (5.0)

Medroxyprogesterone acetate (10)
Methenamine (500)
Methylprednisolone sodium succinate (125)
3-Methylxanthine (500)
Minoxidil (10)
Morphine sulfate (10)
Nalidixic acid (500)
Nicotinic acid (10)
Nitroglycerin (0.4)
Ornade [Smith Kline & French]:
- Chlorpheniramine maleate (8.0)
- Isopropamide (2.5)
- Phenylpropanolamine hydrochloride (50)

Oxazepam (30)
Panothenic acid (10)
Penicillin potassium phenoxymethyl (500)
Phenformin hydrochloride (25)
Phenobarbital (64)

[a]Dose tested in parentheses following each substance is expressed in mg or as noted.

[b]Possible interference with urinary free dopamine assay: λ_{ex} = 320 nm, λ_{em}= 375 nm.

TABLE 2. Substances Exhibiting Moderate Fluorescence During *In Vitro* Testing with the Trihydroxyindole Procedure[a]

Aldactazide [Searle & Co.]:
- Hydrochlorothiazide (25)
- Spironolactone (25)

Chlorthalidone (100)
Cholestyramine resin (4000)
Codeine sulfate (32)
Dantrolene sodium (50)
Digoxin (0.06)
Dimetapp[b,c] [Robbins]:
- Brompheniramine maleate (24)
- Phenylephrine hydrochloride (30)
- Phenylpropanolamine hydrochloride (30)

Diphenhydramine hydrochloride[b,c] (50)
Droxoral [Schering]:
- Dexbrompheniramine maleate (6)
- d-Isoephedrine sulfate (120)

Estrogens, conjugated (0.31)
Erythromycin stearate (500)
Glyceryl guaiacolate (100)
Hydrochlorothiazide (100)
Meprobamate (400)
Propoxyphene hydrochloride (65)
Quinine sulfate[c] (324)
Reserpine (0125)
Tetracycline hydrochloride[c] (250)
Thiamine hydrochloride (50)
Tolbutamide (250)

[a]Dose tested in parentheses following each substance is expressed in mg.

[b]Possible interference with urinary free dopamine assay: λ_{ex} = 320 nm, λ_{em} = 375 nm.

[c]Abnormal blank.

TABLE 3. Substances Exhibiting Strong Fluorescence During *In Vitro* Testing with the Trihydroxyindole Method[a]

Ampicillin (250)
Ascorbic acid[b] (500)
Chlorpromazine hydrochloride[b,c] (25)
Dyazide[c] [Smith Kline & French]:
  Hydrochlorothiazide (25)
  Triamterene (50)
Formaldehyde (1.0)
Isoproterenol hydrochloride[c] (10)
Methenamine mandelate (1000)
Methyldopa (250)
Nitrofurantoin[c] (100)
Phenazopyridine hydrochloride[b,c] (100)
Phenylbutazone[c] (25)
Quinidine sulfate[c] (400)
Riboflavin[b,c] (50)
Rifampin [b,c] (300)
Triamterene[c] (100)

[a]Dose tested in parentheses following each substance is expressed in mg.

[b]Possible interference with urinary free dopamine assay: λ_{ex} = 320 nm, λ_{em} = 375 nm.

[c]Abnormal blank.

TABLE 4. Apparent Catecholamine Values from Pooled Urine in the Presence of Substances Interfering with the Trihydroxyindole Method[a]

Drug(s)[b]	µg Catecholamine[c]	
	Norepinephrine	Epinephrine
Ampicillin (250)	20.4	12.0
Ascorbic acid (500)	13.4	13.0
Chlorpromazine hydrochloride (25)	14.3	4.1
Dyazide[e]	d	15.9
Erythromycin stearate (500)	12.0	13.4
Formaldehyde (1.0)	2.2	d
Isoproterenol hydrochloride (10)	d	24.1
Methenamine mandelate (1000)	5.5	1.7
Methyldopa (500)	49.0	28.0
Phenazopyridine hydrochloride (100)	12.0	d
Phenylbutazone (25)	16.0	9.1
Quinidine sulfate (400)	17.0	7.8
Riboflavin (10)	1.4	0.2
Rifampin (200)	5.6	7.5
Thiamine hydrochloride (50)	12.5	11.7
Triamterene (100)	d	10.4
Ascorbic acid (500) — Methenamine mandelate (1000)	3.2	0.7
Dyazide[e] — Methyldopa (100)	23.0	12.0
Dyazide[e] — Riboflavin (10) — Thiamine Hydrochloride (10)	d	d

[a]Each drug was added to 150 ml of pooled urine adjusted to contain 10 µg each of norepinephrine and epinephrine. Dose tested in mg is in parentheses following each substance.

[b]Drugs studied in combination indicated by bracket.

[c]Adjusted to 100% recovery.

[d]Negative result obtained.

[e]Dyazide [Smith Kline & French]: Hydrochlorothiazide (25), triamterene (50).

DISCUSSION

There are several modes by which drugs may interfere with the trihydroxyindole method. One such mechanism involves drugs such as methyldopa which react to yield fluorescent products. Similar in structure to the biogenic amines, the trihydroxyindole derivatives of these drugs will fluoresce at the emission wavelengths used for norepinephrine and epinephrine determinations yielding spuriously elevated results. Other examples of similar interference include thiamine hydrochloride which may be oxidatively converted to highly fluorescent thiochrome in alkaline solution (29) as seen in Figure 1 and ascorbic acid which, in addition to its antioxidant property, may cause in high concentration formation of fluorescent complexes (30). Both vitamins may cause false elevation of catecholamine excretion as illustrated in Table 4 and Figure 4.

Another apparent mode of interference is provided by formaldehyde, a metabolic breakdown product of methenamine mandelate in urine. Formaldehyde inhibits the recovery of catecholamines during alumina extraction, and may even depress the formation of trihydroxyindole derivatives (24). Such inhibition of extraction onto alumina spuriously decreases apparent catecholamine excretion as seen in Table 4 and Figure 4. A third possible mode of interference is caused by substances such as phenazopyridine hydrochloride and rifampin which in solution form an intense color not removed during alumina extraction, which interferes with fluorescent excitation and emission spectra. These substances exhibit strong absorption near the excitation wavelength maximum (410 nm) used in the trihydroxyindole method, and as seen in Figure 3 for phenazopyridine hydrochloride, will diminish the fluorescent intensity of the trihydroxyindole derivatives in relative proportion to amount of drug present. Such interference usually decreases apparent catecholamine values illustrated by Table 4 and Figure 4.

Substances with native fluorescence such as roboflavin may also interfere. Unfortunately such fluorescence is present in both blank and sample specimens, and determination of catecholamines is altered in unpredictable fashion depending on the relative fluorescence of sample and blank. If the interfering fluorescence contributes to a greater extent in the blank solution than the test solution, a low or negative value for apparent norepinephrine excretion may be obtained (Figure 2). Conversely, if such fluorescence contributes predominately to the test solution an elevated apparent norepinephrine excretion may result. Clearly catecholamine determinations by the trihydroxyindole method in the presence of such interference are meaningless even if they are apparently normal. As an example, riboflavin appears to decrease catecholamine values *in vitro* (Table 4), yet *in vivo* data of Figure 4 indicate no change or perhaps an increase in apparent norepinephrine excretion. Similar inconsistent results from Table 4 are obtained for quinidine sulfate, chlorpromazine hydrochloried and phenylbutazone.

Other substances interfering in similar fashion include triamterene whose emission spectrum is presented in Figure 2 and whose presence in pooled

urine (Table 4) decreased apparent norepinephrine recovery, yet *in vivo* studies of triamterene administration in two individuals resulted in both elevated and decreased apparent norepinephrine excretion. Another example of this type of interference is tetracycline hydrochloride (Figure 1). Presumably other drugs identified in Tables 2 and 3 yielding abnormal blanks may also interfere with the trihydroxyindole method in similar fashion. In most cases, such interference can be identified during fluorometric analysis by observing whether the blank is higher that normally obtained.

Several drug regimens that might be encountered in clinical practice were examined for interference in pooled urine. Each of the three combinations presented in Table 4 might interfere in two different modes with the trihydroxyindole method, and the inconsistent behavior of such interferences is illustrated by the data for Dyazide (Smith Kline & French), and methyldopa (Aldomet, Merck Sharp & Dohme) where norepinephrine concentration is spuriously doubled while epinephrine concentration is apparently unchanged.

The results of this paper should be interpreted with the view that while the kidney may excrete a substance unchanged, it is more likely to be excreted both intact and as a variety of metabolic breakdown products, all of which may vary considerably in their fluorometric activity or interaction with the trihydroxyindole method. This study has examined only a few metabolic products such as 3-methylxanthine and trimethylamine, formed from caffeine and choline chloride, respectively. *In vivo* data as in Figure 4 may also be influenced by variability in drug metabolism among different individuals, by concentration of the substance in urine, and by possible direct action of the drug upon catecholamine metabolism. Further, norepinephrine excretion may be directly influenced by glomerular filtration rate and urine pH (31). Ascorbic acid, riboflavin, and thiamine hydrochloride apparently do not affect urinary free norepinephrine excretion measured by reverse-phase high-pressure liquid chromatography (32).

In summary, this study has expanded upon data previously available in identifying drugs which may spuriously increase or decrease urinary free catecholamine excretion measured by the trihydroxyindole fluorometric method, and has illustrated and discussed several modes of such interference. A careful patient history should be obtained, and inconsistent or abnormal results must be verified by careful examination of the blank and sample fluorescent spectra in question so that drug-induced interferences may be avoided.

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